

Genetic Structure and Distribution of the Colibactin Genomic Island among Members of the Family *Enterobacteriaceae*^{▽†}

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A genomic island encoding the biosynthesis and secretion pathway of putative hybrid nonribosomal peptide-polyketide colibactin has been recently described in *Escherichia coli*. Colibactin acts as a cyclomodulin and blocks the eukaryotic cell cycle. The origin and prevalence of the colibactin island among enterobacteria are unknown. We therefore screened 1,565 isolates of different genera and species related to the *Enterobacteriaceae* by PCR for the presence of this DNA element. The island was detected not only in *E. coli* but also in *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Citrobacter koseri* isolates. It was highly conserved among these species and was always associated with the yersiniabactin determinant. Structural variations between individual strains were only observed in an intergenic region containing variable numbers of tandem repeats. In *E. coli*, the colibactin island was usually restricted to isolates of phylogenetic group B2 and inserted at the *asnW* tRNA locus. Interestingly, in *K. pneumoniae*, *E. aerogenes*, *C. koseri*, and three *E. coli* strains of phylogenetic group B1, the functional colibactin determinant was associated with a genetic element similar to the integrative and conjugative elements ICE*EcI* and ICE*KpI* and to several enterobacterial plasmids. Different *asn* tRNA genes served as chromosomal insertion sites of the ICE-associated colibactin determinant: *asnU* in the three *E. coli* strains of ECOR group B1, and different *asn* tRNA loci in *K. pneumoniae*. The detection of the colibactin genes associated with an ICE-like element in several enterobacteria provides new insights into the spread of this gene cluster and its putative mode of transfer. Our results shed light on the mechanisms of genetic exchange between members of the family *Enterobacteriaceae*.

Horizontal gene transfer between bacteria—even between different species—has been shown to be an important mechanism for exchange of genetic material. This confers a selective advantage to the recipient, e.g., the rapid acquisition of gene clusters coding for pathogenicity or fitness factors. The colibactin genomic island previously discovered in *Escherichia coli* (17, 23) displays several features of a horizontally acquired genomic region: (i) the chromosomal insertion into the *asnW* tRNA locus, (ii) the presence of a P4-like integrase gene, (iii) the presence of flanking 16-bp direct repeats, and (iv) an elevated G+C content relative to the *E. coli* core genome. This genomic island is ~54 kb in size and consists of 20 open reading frames (ORFs), of which 8 code for putative polyketide synthases, nonribosomal peptide synthetases, and hybrids

thereof. Until the discovery of this island the only known non-ribosomal peptide and polyketide/nonribosomal peptide hybrids in *Enterobacteriaceae* have been the iron chelators enterobactin and yersiniabactin, respectively (13, 29). In contrast to these iron chelators, the synthesized hybrid nonribosomal peptide-polyketide colibactin exerts a cytopathic effect on eukaryotic cells in vitro. Upon cocultivation of colibactin island-positive bacteria with eukaryotic cells, DNA double-strand breaks are induced, and the cells are arrested in the G₂ phase of the cell cycle and exhibit megalocytosis and cell death (23). These effects are comparable to the effects of the cyclomodulin cytolethal distending toxin (27, 36), but the biological function of colibactin in vivo is still unknown.

An important mechanism during the evolution of bacteria is horizontal gene transfer. This contributes to the variability of bacterial genomes by enabling bacteria to acquire and incorporate genetic material into their genome, where it may form genomic islands (14). Such genetic material may not always be advantageous to the host and is therefore a genetic and metabolic burden for the bacteria. In this case bacterial genomes tend to lose this excessive information (1, 21). On the other hand, genetic material coding for pathogenicity or fitness fac-

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tors confers a selective advantage to the host. In the case of pathogenic bacteria, this horizontally acquired genetic material may contribute to the colonization and invasion of host tissue. Increased bacterial fitness or pathogenicity promotes the stabilization of the corresponding determinants in the recipient's genome, and the stable integration of horizontally acquired DNA is most frequently connected to a distinct biological function (25).

Until now, the colibactin island was only detected in *E. coli* isolates of phylogenetic lineage ECOR-B2 (23) and was significantly associated with other virulence gene clusters among extraintestinal pathogenic *E. coli* (ExPEC) isolates of ECOR group B2 from diverse clinical sources and with a high virulence potential (18, 19). To learn more about the capacity of dissemination of this genomic island, we investigated its distribution, genetic conservation, and structural organization among members of the *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 640 enterohemorrhagic *E. coli* strains, 205 extraintestinal pathogenic *E. coli* isolates, 135 *E. coli* fecal isolates from healthy volunteers, and 56 *E. coli* isolates from diverse sources were included in the present study. Furthermore, 287 *Klebsiella* isolates were tested. This group was composed of 141 clinical *Klebsiella pneumoniae* isolates from France and Germany; 103 *K. oxytoca* strains (including a well-characterized collection from Sweden) (38); 14 *K. terrigena*, 11 *K. planticola*, 1 *K. edwardsii*, 1 *K. rhinoscleromatis*, and 1 *K. ozaenae* isolate; and 15 *Klebsiella* strains that were not further typed. A total of 114 *Salmonella enterica* isolates from different subspecies and serovars, including the SARC collection and 13 yersiniabactin-positive isolates of subspecies III and VI (26), and 40 *Yersinia* strains (including multiple *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*, and *Y. kristensii* isolates) were also tested for the presence of the colibactin island. Also, 33 *Proteus* strains (including several *P. mirabilis*, *P. morgani*, and *P. penneri* isolates), 17 *Serratia* strains, 12 *Enterobacter* strains (including 11 *E. aerogenes* and 1 *E. cloacae* strain), and 10 *Shigella* isolates (including 4 *S. dysenteriae*, 2 *S. flexneri*, 2 *S. sonnei*, and 2 *S. boydii* strains) were also included into the present study. In addition to the sequenced *Citrobacter koseri* strain ATCC BAA-895, four *C. freundii* isolates were screened for the colibactin gene cluster. We also examined two *Photobacterium luminescens*, three *Xenorhabdus* spp., two *Pantoea agglomerans*, one *Providencia* sp., and one *Erwinia herbicola* isolate, as well as one *Escherichia hermannii* and one *E. fergusonii* isolate, for the presence of the colibactin island. *E. coli* JM109 [*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) $e14^-$ (Δ *mcrA*) *supE44 relA1* (Δ (*lac-proAB*)/F' (*traD36 proAB⁺ lacI^a lacZ* Δ M15))] was used to prepare competent cells. A deletion mutant of colibactin-producing *E. coli* strain Nissle 1917 that carries a 29.5-kb deletion comprising the yersiniabactin determinant (*ybtS-fyuA*) was used to investigate the dependence of colibactin expression on the presence of the yersiniabactin determinant. Depending on the experiments, the strains were grown in Luria broth or on Mueller-Hinton agar at 37°C for 18 to 24 h.

Detection of the colibactin island in different isolates. The presence of the colibactin island among enterobacterial isolates was determined by PCR using primers published previously (23). The initial PCR screening was performed with the primers ORF 1907-1908 and ORF 1919-1920 using *E. coli* strain Nissle 1917 genomic DNA as a positive control. Intra-colibactin island-specific PCRs were then performed using the primers ORF 1911-1912, ORF 1913-1914, ORF 1915-1918, and ORF 1920-1922. The primers *asnW*-PALleftend and *asnW*-PALrightend specific for the left and right junctions of the colibactin island, respectively, were also used.

MLST. The allocation of the *E. coli* isolates to different clonal lineages was performed as described elsewhere (<http://mlst.ucc.ie>). Sequence types (STs) were assigned using the *E. coli* multilocus sequence typing (MLST) database hosted at the University College Cork, Cork, Ireland (<http://mlst.ucc.ie>). Information regarding new STs was deposited at the *E. coli* MLST database.

Sequencing of the *clbA* gene and the left junction of the colibactin island. According to the published sequence of the colibactin island (accession no. AM229678), primers ClbA 1F and ClbA 1R (see Table S1 in the supplemental material) were used to amplify a 735-bp fragment from the *K. pneumoniae* strain CF1 genomic DNA. Amplification was performed using Platinum Taq DNA

polymerase (Invitrogen) according to the manufacturer's instructions. The PCR fragment was purified with the NucleoSpin Extract II kit from Macherey-Nagel. The primers ClbA 1F and *asnW*-PALleftend2 (see Table S1 in the supplemental material) were used to amplify a 3,303-bp fragment from strain CF1. Amplification was performed by using the Expand Long Template PCR system (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. The PCR fragment was purified, ligated into the pDrive cloning vector (Qiagen PCR cloning kit), and transformed into JM109 competent cells. Plasmid DNA was isolated by using NucleoSpin plasmid (Macherey-Nagel), and the left-hand junction of the colibactin determinant was sequenced by using the universal primers SP6 and T7 promoter. Sequences were commercially obtained from Cogenics (Meylan, France). Sequence homology was analyzed by using the BLAST 2.0 search algorithm at the National Center for Biotechnology Information (2). The sequence has been submitted to the National Center for Biotechnology Information database (accession no. FJ899134). Putative ORFs were identified by using Vector NTI (InforMax, Oxford, United Kingdom) and Artemis (30). The Artemis Comparison Tool was used as a DNA sequence comparison viewer (10).

Characterization of the colibactin determinant left-hand sequence context. The left-hand junction of the colibactin gene cluster in other colibactin-positive *K. pneumoniae* and *E. aerogenes* strains was amplified with the primers *pksp* F and *asnW*-PALleftend2 (see Table S1 in the supplemental material). In addition, ICEKp1-like elements adjacent to the colibactin determinant were searched among the colibactin-positive *K. pneumoniae* and *E. aerogenes* strains by PCR using the primer *fyuA* (5' region) and *virB1* (3' region) genes as described by Lin et al. (20). Primers outside the middle region (HPI 3'-F and *virB1*-F inverse) and primers derived from the left (orf3-R) and right (orf16-F) parts of the middle region were also used. Analysis of the *C. koseri* strain ATCC BAA-895 genome sequence (accession no. CP000822) indicated the insertion of a DNA region with similarity to ICEEcl1 and ICEKp1 between the *clbQ* gene and the *intP4* homolog of the colibactin gene cluster. To screen this 85-kb DNA region between the colibactin biosynthesis determinant and the next *asn* tRNA gene of *C. koseri* strain ATCC BAA-895 in more detail in other enterobacterial isolates, 102 primer pairs (see Table S1 in the supplemental material) were designed to screen for the presence of this genomic region by overlapping PCRs.

Determination of chromosomal integration sites. To determine the chromosomal integration site of the colibactin island in *Klebsiella* and *E. coli* strains, restriction fragments of genomic DNA were sequenced by inverse PCR (24). Alternatively, genomic DNA was directly sequenced by primer walking starting from the regions with known nucleotide sequences. In case of direct sequencing of genomic DNA, a Qiagen genomic DNA isolation kit was used to isolate the genomic DNA. A total of 6 μ g of DNA was used as a template for direct sequencing of genomic DNA using an ABI 310 sequencer. Primer concentrations ranged from 0.5 to 1 μ M. For destabilization of DNA secondary structures, betaine was added to a concentration of 0.25 M.

Analysis of the variable-number tandem repeat (VNTR) region. The number of variable repeats between *clbB* and *clbR* was determined by DNA sequence analysis upon amplification of the corresponding genomic region by PCR using the primers *varregionPKS*.for and *varregionPKS*.rev. The resulting PCR product was then purified by using a QIAquick PCR purification kit (Qiagen) and directly used as a template for DNA sequencing with the primer pair *varregionPKS*.seq.for and *varregionPKS*.seq.rev using an ABI 310 sequencer.

Cell culture, bacterial infections, γ H2AX staining, and cell cycle analysis. For bacterial infections, overnight Luria broth cultures of bacteria were diluted in interaction medium (Dulbecco modified Eagle medium, 5% fetal calf serum, 25 mM HEPES [Invitrogen]), and ~50% confluent HeLa cell cultures (ATCC CCL2) were infected at a multiplicity of infection of 100. Cells were washed three to six times 4 h after inoculation and incubated in Dulbecco modified Eagle medium, 10% fetal calf serum, and 200 μ g of gentamicin or 500 μ g of streptomycin/ml until analysis as described previously (23). Briefly, for γ H2AX staining the cells were fixed in 95% methanol-5% acetic acid and then incubated with anti-phospho(Ser139)-H2AX antibodies (JBW301; Upstate), followed by fluorescein isothiocyanate-conjugated secondary antibodies. DNA was stained with TO-PRO-3 (Invitrogen), and images were acquired with an Olympus IX70 laser scanning confocal microscope, objective PlanApo $\times 60$ (NA 1.4), and Fluoview FV500 software, the confocal aperture being set to achieve a z optical thickness of ~0.5 μ m. For cell cycle analysis, nuclear suspensions were made directly from adherent cells with 0.1% sodium citrate, 1% NP-40, 50 μ g of propidium iodide/ml, and 250 μ g of RNase/ml. Nuclei DNA content data were acquired with a FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (Tree Star).

TABLE 1. Presence of colibactin island in different members of the family *Enterobacteriaceae* and related genera

Genus	Species	Type ^a	No. of isolates tested	No. of colibactin island-positive isolates	Prevalence (%)
<i>Escherichia</i>	<i>coli</i>	ExPEC	205	76	37.1
		Fecal isolates from physiological flora	142	28	19.7
		EHEC, EPEC, other pathotypes	689	0	0
	<i>hermannii</i>		1	0	0
	<i>fergusonii</i>		1	0	0
<i>Citrobacter</i>	<i>koseri</i>		1	1	100
	<i>freundii</i>		4	0	0
<i>Klebsiella</i>	<i>pneumoniae</i>		141	5	3.5
	<i>oxytoca</i>		103	0	0
	<i>terrigena</i>		14	0	0
	<i>planticola</i>		11	0	0
	<i>edwardsii</i>		1	0	0
	<i>ozaenae</i>		1	0	0
	<i>rhinoscleromatis</i>		1	0	0
	spp.		15	0	0
<i>Enterobacter</i>	<i>aerogenes</i>		11	3	27.3
	<i>cloacae</i>		1	0	0
<i>Salmonella</i>	<i>enterica</i>		114	0	0
<i>Shigella</i>	spp.		10	0	0
<i>Yersinia</i>	spp.		40	0	0
<i>Proteus</i>	spp.		33	0	0
<i>Serratia</i>	spp.		17	0	0
<i>Photobacterium</i>	<i>luminescens</i>		2	0	0
<i>Xenorhabdus</i>	spp.		3	0	0
<i>Providencia</i>	spp.		1	0	0
<i>Pantoea</i>	<i>agglomerans</i>		2	0	0
<i>Erwinia</i>	<i>herbicola</i>		1	0	0

^a EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*.

RESULTS

Prevalence of the colibactin island among different genera and species of the *Enterobacteriaceae*. A total of 1,565 bacterial isolates of different enterobacterial genera and species (Table 1) were screened by PCR (Fig. 1) for the presence of the colibactin determinant.

In *E. coli*, 104 of 1,092 (9.5%) isolates tested harbored the island, although the majority (73.1%) of the colibactin-positive *E. coli* strains were clinical ExPEC, and 26.9% were commensal *E. coli* strains isolated from healthy volunteers. In contrast, none of the 689 intestinal pathogenic *E. coli* strains tested harbored the colibactin island. Interestingly, this gene cluster was only present in *E. coli* strains of phylogenetic lineage B2,

except three extended-spectrum β -lactamase-positive *E. coli* O153:H31 isolates of ECOR group B1 (U12633, U19010, and U15156). According to MLST, these three strains belong to ST 101.

The colibactin island was detected not only in *E. coli* but also in 5 of 141 (3.5%) *K. pneumoniae* strains (strains CF1, CF44, 41, Kp52145, and SB3431), 3 of 11 (27.3%) *E. aerogenes* strains (strains 20, 50, and 64), and a *C. koseri* isolate (ATCC BAA-895). These colibactin island-positive strains are clinical extraintestinal pathogenic isolates, which are frequently resistant to several antibiotics.

The functionality of the polyketide gene cluster in *K. pneumoniae*, *E. aerogenes*, *E. coli* B1, and *C. koseri* strains was

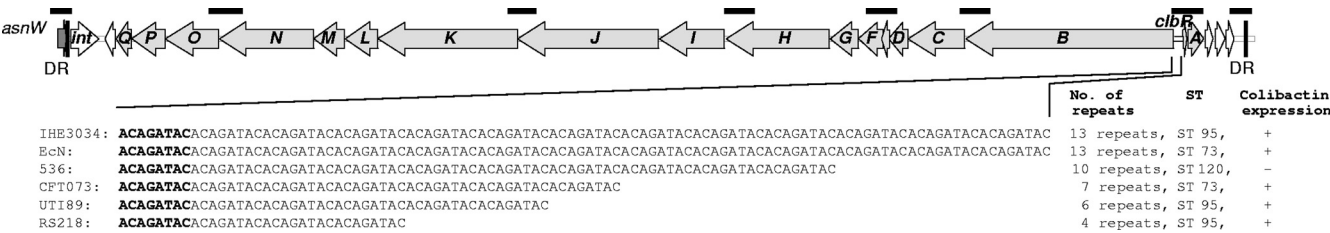


FIG. 1. Genetic structure of the colibactin island of *E. coli* strain IHE3034. This genomic island is flanked by direct repeats (DR) and is inserted at *asnW* into the bacterial chromosome. The colibactin biosynthesis gene cluster is indicated by gray arrows. DNA regions used for PCR screening are indicated by black lines above the gene cluster. The VNTR located between *clbB* and *clbR* in different *E. coli* isolates differs in size. The number of repeats, the ST of the host strain, and the colibactin phenotype is given. EcN, *E. coli* strain Nissle 1917.

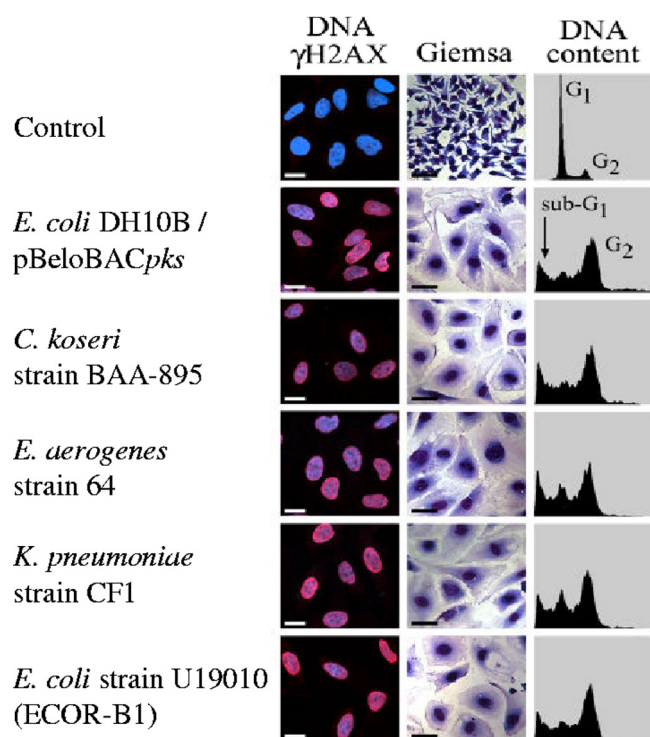


FIG. 2. Phenotypic analysis of colibactin expression in different enterobacteria. HeLa cells were infected for 4 h with *C. koseri*, *K. pneumoniae*, *E. aerogenes*, and ECOR-B1 *E. coli* representative isolates or with DH10B pBACpks as a positive control (23). In the left panels, histone H2AX Ser139 phosphorylation (γ H2AX) indicative of DNA double-strand breaks was assayed by confocal immunofluorescence at 4 h after infection. DNA and γ H2AX are pseudocolored in blue and red, respectively. Bars, 20 μ m. In the middle panels, the cell morphology was observed after Giemsa staining at 72 h after infection. Bars, 50 μ m. In the right panels, G2 cell cycle arrest and increased sub-G1 cell populations (cell death) after DNA damage were assayed by flow cytometry at 72 h after infection. Similar results were obtained with all other colibactin island-positive *C. koseri*, *K. pneumoniae*, *E. aerogenes*, and ECOR-B1 *E. coli* isolates (data not shown).

confirmed on HeLa cell cultures (Fig. 2). The cells exposed to each colibactin island-positive strain exhibited histone H2AX phosphorylation, cell body, and nucleus enlargement (megakaryocytosis), G2 cell cycle arrest and DNA fragmentation (sub-G1 peak indicative of cell death), findings indicating that all colibactin-producing enterobacteria induced host DNA double-strand breaks similarly to colibactin-positive *E. coli* strains of phylogenetic group B2 (23). Thus, the cytopathic phenotype associated with the colibactin island was fully conserved in the different enterobacterial isolates.

A positive correlation was observed between the presence of the colibactin determinant and the high pathogenicity island (HPI) coding for the siderophore system yersiniabactin (9): all colibactin-positive strains were also yersiniabactin positive. Nevertheless, the presence of the HPI was not always associated in *E. coli*, *K. pneumoniae*, and *E. aerogenes* with that of the colibactin genes. Similarly, in *S. enterica* isolates of subspecies IIIa, IIIb, and VI that have been previously described to be HPI positive (26), the colibactin gene cluster could not be detected by PCR screening. Expression of colibactin was independent of yersiniabactin expression. This was corroborated by

the fact that colibactin could be expressed in yersiniabactin-negative *E. coli* K-12 strain DH10B (23) and also by the comparison of the cytopathic effect of strain Nissle 1917 and its HPI-negative mutant since both strains induced the cytopathic effect characteristic for colibactin expression (data not shown).

Size of the VNTR of the colibactin island. A DNA sequence comparison of the available genome sequences of *E. coli* strains 536 (accession no. NC_008253), UTI89 (NC_007946), CFT073 (NC_004431) and the colibactin island of *E. coli* isolate IHE3034 (accession no. AM229678), as well as sample sequencing of *clbA* of *K. pneumoniae* strain CF1, indicated that the colibactin biosynthesis and secretion determinant was generally highly conserved (>98% nucleotide sequence identity) in the different enterobacterial isolates. As an exception, a VNTR between *clbB* and *clbR* exhibited marked size variations in individual isolates (Fig. 1). DNA sequence analysis of this region in 99 different colibactin-positive extraintestinal pathogenic or commensal *E. coli* and *Klebsiella* isolates revealed that the repeat region comprises between 2 and 20 repeats of the octanucleotide sequence 5'-ACAGATAC-3' and thus typically represents a VNTR (22). The most prevalent variants of the repeat region consist of 6 to 10 repeats. In the case of the *C. koseri* isolate ATCC BAA-895, 11 repeat units were found. VNTRs have been used as DNA markers for molecular typing of several bacterial species (11). A correlation between (i) the size of the region, (ii) even or odd numbers of repeats, (iii) relatedness of strains (as determined by MLST), (iv) the phenotype, and (vi) colibactin activity could not be detected thus far (data not shown).

Sequence context of the tRNA-proximal junction of the colibactin determinant. In ECOR group B2 *E. coli*, the colibactin gene cluster was found to be integrated at the *asnW* tRNA gene. To characterize the genetic context of the colibactin gene cluster in *K. pneumoniae*, a 3,303-bp PCR fragment covering the left junction was amplified in strain CF1 with the primers ClbA 1F and *asnW*-PALleftend2 and subsequently sequenced (accession no. FJ899134). Database comparison of this sequence revealed a 3,243-bp fragment with 99% identity to the genome sequence of *C. koseri* ATCC BAA-895 (accession no. NC_009792). In this fragment, the *E. coli* IHE3034 1,841-bp region upstream of ORF2 comprising the *asnW* tRNA locus and the P4-like integrase-encoding gene *intP4* (accession no. AM229678) was replaced in *K. pneumoniae* CF1 and *C. koseri* ATCC BAA-895 by a 1,174-bp DNA stretch without significant homology, followed by a 1,134-bp region with 83% identity to ORFs coding for the putative MobC and MobB proteins of pCRY of *Yersinia pestis* biovar Microtus strain 91001 (NC_005814) and pMET-1 of *K. pneumoniae* FC1 (EU383016), respectively (Fig. 3 and 4). The sequence context of the putative *mobC* (CKO_00879) and *mobB* (CKO_00880) genes in *C. koseri* BAA-895 (CKO_00917 to CKO_00879) exhibits 91 to 98% nucleotide identity to the *asn* tRNA gene-associated integrative and conjugative elements ICEEc1 (AY233333) and ICEKp1 (AB298504), respectively.

Detection of the ICE element and identification of its chromosomal insertion site in enterobacterial isolates. Colibactin-positive enterobacterial isolates were screened by PCR with 102 primer pairs (see Table S1 in the supplemental material) for the presence of the 85-kb chromosomal segment of *C. koseri* BAA-895 that covers the ICE-like region located be-

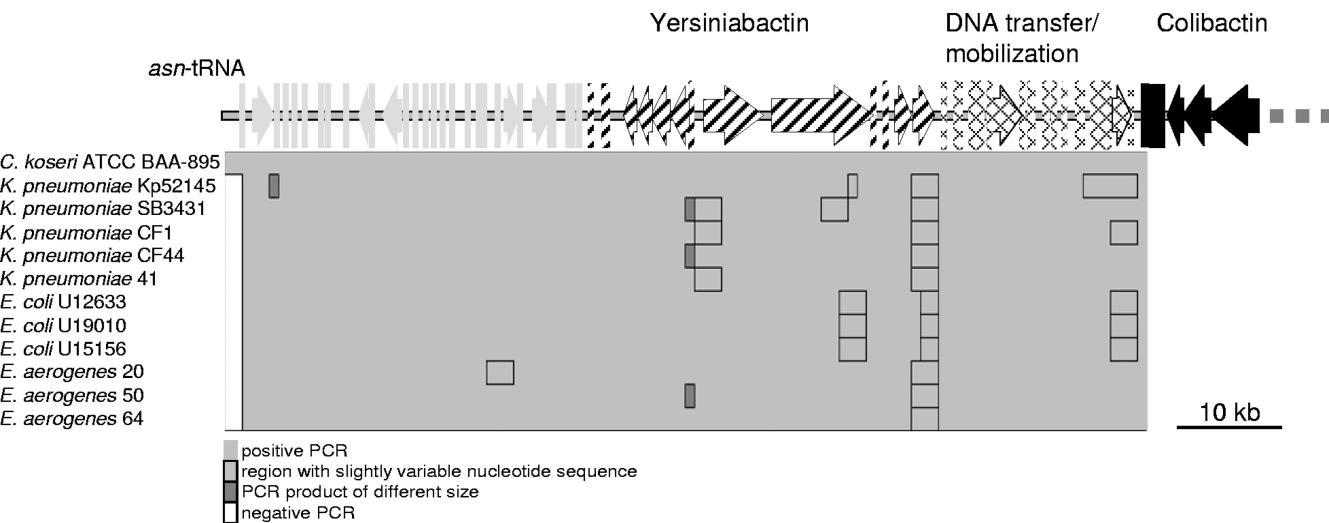


FIG. 3. Association of an ICE-like DNA region with the colibactin determinant in different members of the family *Enterobacteriaceae*. The tRNA-proximal sequence context of the integrative element comprising the colibactin gene cluster in *C. koseri* ATCC strain BAA-895 was screened by overlapping PCR in different colibactin-positive enterobacterial strains. The results of the PCR screening are summarized in the box below the genetic map. Homologous DNA regions are indicated in light gray. Regions with minor nucleotide sequence differences are boxed. Variable DNA stretches representing insertions or deletions are indicated in dark gray, and DNA regions that could not be amplified by PCR are shown in white.

tween the colibactin determinant to the *asn* tRNA gene (Fig. 3). Interestingly, the colibactin gene cluster present in *K. pneumoniae*, *E. aerogenes*, and ECOR-B1 *E. coli* isolates was part of an ICE similar to that of *C. koseri* BAA-895. The genetic organization of the ICE-like element containing the colibactin genes in *C. koseri* was structurally conserved in these isolates (Fig. 3). Certain regions including genes involved in DNA transfer and mobilization and yersiniabactin biosynthesis, as well as some hypothetical ORFs further upstream of the

yersiniabactin determinant, exhibited minor DNA sequence variation (Fig. 3 and Table 2), as evidenced by the necessity to amplify these regions with different combinations of individual primers located further up- or downstream (Fig. 3). Additional PCR screenings with the primers *fyuA*-F, *fyuA*-R, *virB1*-F, *virB1*-R, *HPI* 3'-F, *virB1*-F inverse, *orf3*-R, and *orf16*-f (see Table S1 in the supplemental material) designed on the basis of the ICE*Kp1* nucleotide sequence further supported our results (data not shown). One region, located within the yersinia-

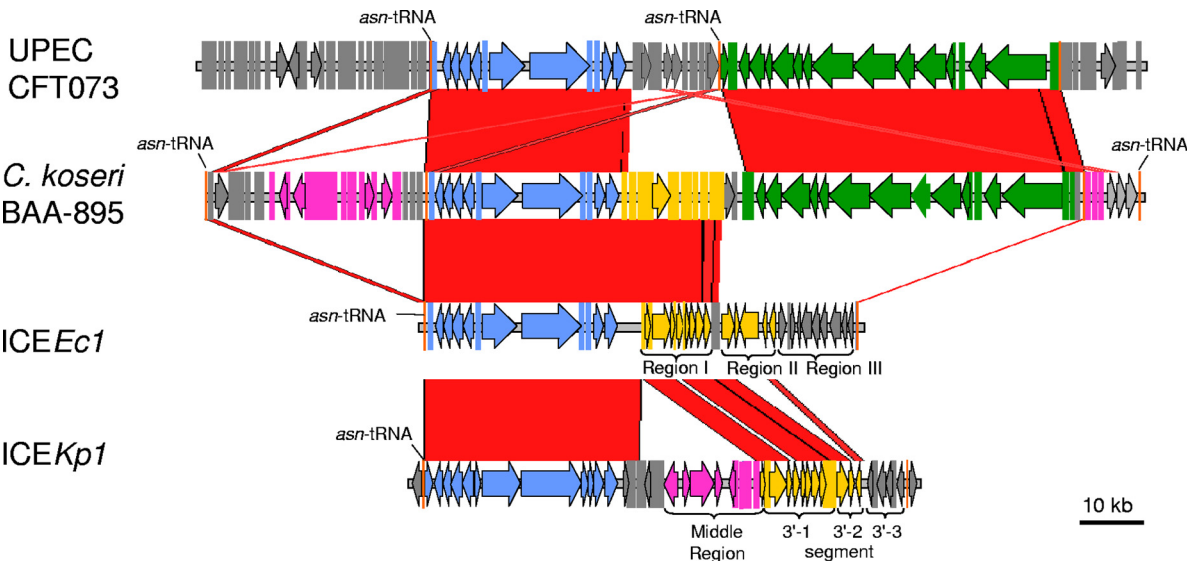


FIG. 4. Genetic structure of the sequence context of the colibactin determinant in *C. koseri* strain ATCC BAA 895 and its comparison to other corresponding enterobacterial genome regions. Nucleotide sequence homology between different DNA regions is indicated by red areas between the corresponding genetic maps. Homologous regions are indicated by different colors according to their function: the yersiniabactin gene cluster is represented in blue, the DNA transfer and mobilization region is represented in yellow, the colibactin determinant is represented in green, and pLVK-related DNA regions are represented in pink. Direct repeat regions are indicated in orange.

TABLE 2. Variable regions of ICE*ck1* in *K. pneumoniae*, *E. aerogenes*, and *E. coli*

Chromosomal region (bp) in <i>C. koseri</i> ATCC BAA-895	ORF	Encoded protein
871749–872718	CKO_00880	Putative mobilization protein MobB
873448–874420	CKO_00882	Hypothetical protein
	CKO_00883	Hypothetical protein
	CKO_00884	Hypothetical protein, similar to YggA-like protein
	CKO_00885	Hypothetical protein
875140–876138	CKO_00886	Type IV secretory pathway; VirB11 component
	CKO_00887	Hypothetical protein
	CKO_00888	Hypothetical protein
	CKO_00889	Type IV secretory pathway; VirB10 component
889228–891838	CKO_00905	FyuA
	CKO_00906	YbtE
896483–897510	CKO_00909	Irp1
909271–910313	CKO_00911	Irp2
910971–912006	CKO_00912	YbtA
	CKO_00913	YbtP
926069–927069	CKO_00927	Hypothetical protein, Fe ²⁺ transport system protein
	CKO_00928	Hypothetical protein, thioredoxin transferase
949741–950779	CKO_00952	Hypothetical protein, predicted phosphatase, insertion of insertion sequence IS2 at base 950500 (1,330 bp)

bactin determinant of *K. pneumoniae* Kp52145, differed in size from the *C. koseri* sequence because of a 331-bp deletion of the *C. koseri* chromosomal region from positions 875542 to 875873 that affected ORFs CKO_00887 to CKO_00889 coding putatively for hypothetical proteins. In *K. pneumoniae* SB3431 and CF44 and in *E. aerogenes* strain 50, a 100- to 200-bp insertion was detected at positions 910971 to 912006 of the *C. koseri* chromosome. Finally, a 1,330-bp insertion sequence (IS2), integrated at base 950500 of the reference *C. koseri* ATCC BAA895 genome, was found to interrupt ORF CKO_00952 (Fig. 3 and Table 2).

Inverse PCR and direct primer walking on the chromosome was performed to identify the ICE chromosomal insertion site in all of the colibactin-positive *E. coli* and *K. pneumoniae* isolates. Whereas the colibactin island has been exclusively detected in the *asnW* tRNA locus in ExPEC and commensal *E. coli*, the ICE-like region containing the colibactin genes was inserted at the *asnU* locus in the three *E. coli* strains of ECOR group B1. In *K. pneumoniae*, the integrative element was inserted into different *asn* tRNA genes: in *asn-1* for strains CF1 and 41, in *asn-2* for strain Kp52145, and in *asn-3* for strain SB3431. In *K. pneumoniae* CF44 the chromosomal insertion site of the ICE-like element differs from that found in the other *K. pneumoniae* isolates but is not adjacent to the *asn-4* tRNA gene.

Comparative analysis of the ICE-like element in *C. koseri* and other related enterobacterial sequences. In *C. koseri*, the putative ICE comprising the colibactin determinant was chromosomally located in close vicinity to asparagine (*asn*) tRNA genes (between CKO_0838 and CKO_0953) and was flanked

by 17-bp direct repeats. One 17-bp direct repeat sequence was found upstream of the integrase gene (CKO_00917) of the yersiniabactin determinant, and another direct repeat was downstream of CKO_0855 at the right end of the colibactin gene cluster.

The yersiniabactin gene cluster located in this ICE-like element showed 98% DNA sequence identity to its counterparts in ICE*Ec1* and ICE*Kp1*. In the latter two elements, this siderophore determinant is followed by DNA sequences (region I_{ICE}*Ec1* and II_{ICE}*Ec1* or segments 3'-1_{ICE}*Kp1* and 3'-2_{ICE}*Kp1*, respectively) that are involved in conjugative transfer and mating-pair formation, as well as by a third variable segment (region III_{ICE}*Ec1* or segment 3'-3_{ICE}*Kp1*) that comprises hypothetical genes (20, 33).

Overall, the right-hand region of the yersiniabactin gene cluster of *C. koseri* ATCC BAA895 exhibited a similar structural organization of the segments 3'-1 (*virB1-virB11*), 3'-2 (*mobB*, *mobC*, and a putative origin of transfer [*oriT*] located upstream of *mobB* at positions 872973 to 872893 on the *C. koseri* chromosome), and 3'-3 (colibactin gene cluster) (Fig. 4). On the other hand, comparative analysis of the different ICEs revealed a modular organization with homologous regions (the yersiniabactin gene cluster and the genes involved in mating-pair formation and DNA mobilization) separated by variable DNA stretches: in ICE*Kp1*, the yersiniabactin determinant is followed by a so-called "middle region" that is similar to a portion of the large virulence plasmid pLVPK of *K. pneumoniae* CG43 (NC_005249). In *C. koseri*, however, fragments of this middle region lacking the virulence-associated genes *vagC-vagD*, *iroN-iroB-iroC-iroD*, and *mmpA* are located at both ends of the ICE-like element and exhibit 76 to 100% nucleotide identity to parts of plasmid pLVPK (Fig. 4). The conserved region required for mating-pair formation and DNA mobilization is identical to a region of the *K. pneumoniae* multiresistance plasmid pMET1 (87 to 93%), to a region of the *Y. pestis* plasmid pCRY (88 to 91%), and to the *Enterobacter sakazakii* plasmid pESA2 (77 to 79%). In the case of *C. koseri* ATCC strain BAA895, the colibactin-positive *K. pneumoniae*, *E. aerogenes*, and *E. coli* ECOR-B1 isolates, this region is then followed by the colibactin determinant, whereas other variable DNA sequences can be found at the right-hand end of this region in ICE*Kp1* and ICE*Ec1* (Fig. 4).

DISCUSSION

In this study, we report on the prevalence, genetic structure, and sequence context of the colibactin island in *Enterobacteriaceae*. We show that this polyketide determinant is present on the chromosome of various coliform enterobacterial species such as *E. coli*, *C. koseri*, *K. pneumoniae*, and *E. aerogenes* and that its presence is associated with that of the yersiniabactin gene cluster, which also encodes a polyketide (28). The distribution of the colibactin determinant in *Enterobacteriaceae* resembles that of the HPI coding for yersiniabactin as described previously (3, 31) and thus further corroborates our observation that the colibactin gene cluster is linked to the yersiniabactin determinant. In analogy to the HPI, the colibactin genes seem to be more widely distributed among certain lineages of *E. coli* than among other coliform enterobacteria: the colibactin genes have been exclusively detected in *E. coli* isolates of

phylogenetic lineage ECOR-B2 thus far (23). This island was also found to be significantly associated with multiple other virulence gene clusters, including the HPI among ExPEC, as well as with high virulence potential (18, 19). Furthermore, in *E. coli* the colibactin gene cluster, together with other ExPEC virulence genes, has been reported to be more frequently detected in mucosa-associated isolates of patients with colon cancer than in strains isolated from healthy individuals (5). The higher prevalence of colibactin genes in pathogenic isolates relative to commensal variants is probably generally true in enterobacteria since the other colibactin-positive enterobacterial strains described in the present study are also clinical pathogenic isolates. Whether colibactin expression contributes to an increased pathogenic potential remains to be elucidated. On the other hand, the colibactin determinant has been detected in probiotic *E. coli* strain Nissle 1917 (15) but not in probiotic *E. coli* strain O83:K24:H31 (Colinfant) (16). This observation indicates that colibactin expression is not a prerequisite for efficient intestinal colonization and the increased competitiveness that may be associated with the probiotic character of these strains.

Another striking similarity between the colibactin and yersiniabactin polyketide determinants is their localization within two different forms of genetic elements usually associated with *asn* tRNA genes. Thus far, these polyketide determinants have been described as part of individual genomic or pathogenicity island, in which they are, together with a P4-like bacteriophage integrase gene, flanked by direct repeats (6, 23). Alternatively, both polyketide gene clusters together can be part of a genomic region with similarity to integrative and conjugative elements that comprises additional genes coding for a conjugative DNA transfer and mobilization system (7, 8). These integrative elements can transfer the yersiniabactin and probably also the colibactin determinants to new recipients, and their transfer activity and plasticity are thus crucial for the genetic diversity observed at *asn* tRNA loci in yersiniabactin- and colibactin-positive enterobacteria. The presence of a second yersiniabactin determinant (CKO_01243 to CKO_01253) located in a different genomic context ~243 kb away from the ICE-like element harboring the yersiniabactin and colibactin genes in the *C. koseri* strain BAA-895 genome also mirrors the considerable genome plasticity involved in the spread of this siderophore determinant. Interestingly, this copy of the yersiniabactin gene cluster lacks an integrase gene and the flanking 17-bp direct repeats.

Based on amino acid sequence comparison between integrases encoded by different integrative elements including genomic islands, integrons, ICEs, conjugative transposons and bacteriophages, it has been recently suggested that genomic islands form a distinct group of ancient genetic elements unrelated to the other integrative elements (4). The analysis of the integrase proteins CKO_0917 and CKO_0953 encoded by the integrative element in *C. koseri* comprising the colibactin determinant indicates that they are 95% identical to each other. In addition, they are highly similar to the integrases (IntG) of other archetypal genomic islands but not to those of well-described ICEs (IntC). Similarly, the *asn* tRNA gene-associated integrase gene of the HPI in ECOR-B2 *E. coli* isolates codes for an IntG-like integrase (4). The ICEEc1-encoded integrase has been shown to differ from other HPI-

encoded integrases, and Boyd et al. hypothesized that an ICE used the same chromosomal insertion site as the HPI in this strain, thereby displacing the HPI integrase (4).

The similarity between the integrative element in *C. koseri* and other chromosomal integrative elements such as the ICEEc1 or ICEKp1 (20, 33, 34), as well as their partial homology to regions of enterobacterial plasmids such as pCRY (35), pMET1 (34), pESA2 (CP000784), and pLVPK (12) involved in DNA transfer and mobilization indicates that chromosomally and plasmid-encoded virulence and resistance-associated gene clusters can efficiently recombine. Our results also demonstrate that active genetic exchanges occur between different members of the *Enterobacteriaceae* (Fig. 4). It can be assumed that the HPI and the colibactin island, as they have been described in the archetypal ECOR-B2 strains, may have evolved from an integrative element by DNA rearrangements and subsequent loss of ICE regions between the colibactin and yersiniabactin determinants. This could be in line with a selective pressure exerted on the yersiniabactin and colibactin determinants, a finding indicative of a role in bacterial fitness.

ICEEc1 and ICEKp1 can excise themselves from the chromosome and, upon circularization and subsequent transfer into a suitable host, integrate into *asn* tRNA loci (20, 33). The colibactin determinant might spread among different enterobacteria by such a mechanism. Our finding that this integrative element is found inserted at different *asn* tRNA genes in *E. coli*, *K. pneumoniae*, and *E. aerogenes* supports this hypothesis. These *asn*-tRNA locus-associated genetic elements seem to represent vehicles of variable modular composition that can be transmitted among different members of the *Enterobacteriaceae*. This group of enterobacterial ICE-like genetic elements seems to be responsible for the dissemination of the yersiniabactin gene cluster and other DNA regions, represented by different region 3 (ICEEc1) and segment 3'-3 (ICEKp1) variants or the colibactin determinant, once they are colocalized. Comparison of the average G+C content of the genomes of *C. koseri* ATCC BAA_895 (53% G+C), *K. pneumoniae* MGH 78578 (57% G+C), and *E. coli* (50% G+C) to that of the colibactin gene cluster (53% G+C) as we describe here suggests that this polyketide determinant may originate from *C. koseri*. It is, nevertheless, still unclear from which source *C. koseri* acquired the colibactin gene cluster.

Interestingly, the four *asn* tRNA genes in *E. coli* and thus the yersiniabactin and colibactin gene clusters are located within a "hot spot of phylogenetic incongruence" that is characterized by a high frequency of DNA insertion and recombination (37). It has also been recently suggested that the HPI has been propagated in *E. coli* by homologous recombination after a unique and recent chromosomal integration event (32). The correlation between the presence of the yersiniabactin and colibactin determinants together with their genetic linkage implies that the high conservation of both polyketide gene clusters in *E. coli* is a result of this unique and recent acquisition followed by their dissemination via homologous recombination. Although we still miss the origin of the colibactin determinant, our findings demonstrate that coliform enterobacteria acquired this gene cluster recently together with the HPI and that mobile DNA elements such as plasmids and ICE-like elements are involved in its horizontal transfer.

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